A novel *P{lacW}* insertion in the *CG18135* gene of *Drosophila melanogaster* is linked to pupal lethality and gene overexpression in adult males

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Abstract

The *CG18135* gene in *Drosophila melanogaster* has documented functions in myosin filament binding and glycerophospholipids catabolism. In this study we used flies harboring a novel *P{lacW}* insertion in the *CG18135* gene (*CG18135*$^{P\{lacW\}CG18135}$) in order to assess mutant embryos and pupae viability, and to investigate the expression profile of *CG18135*. Our results show for the first time that *CG18135*$^{P\{lacW\}CG18135}$ homozygotes lethality is strongly associated with the pupal developmental stage. The gene expression experiment confirms previously identified *CG18135* upregulation in adult males compared to adult females, and revels an unexpected upregulation in heterozygous mutants compared to wild-type flies.

Key words: *CG18135*, *GPCPD1*, *Drosophila melanogaster*, viability, gene expression

Introduction

Drosophila melanogaster has been studied for over a century, being the definitive model organism in genetics, biochemistry, molecular biology and bioinformatics. Since its first genome sequencing in 2000 (Adams *et al.*, 2000), a number of computed genes (CG) have not been adequately characterized and annotated. One such gene is *CG18135* which was identified in 2004 via computational techniques (FlyBase Curators *et al.*, 2004).

Located on the left arm of the chromosome 3 (3L), on the (-) strand, *CG18135* is known to encode for five transcripts which are translated in proteins having between 636 and 770 amino acids. *CG18135* sequence is conserved in higher eukaryotes such as mammals, birds, fish, reptiles, but also in invertebrates and yeasts. Whilst the function of the *CG18135* gene was not previously directly studied in *D. melanogaster*, the associated protein was shown to facilitate myosin filament binding during embryogenesis via interaction with the Myo10A protein at the plasma membrane level (Liu *et al.*, 2008) and to participate in glycerophospholipids catabolism (Gaudet *et al.*, 2011). *CG18135* gene is orthologous to human *glycerophosphocholine phosphodiesterase 1* (*GPCPD1*).

CG18135 has been documented to participate in embryogenesis and in all developmental stages in *D. melanogaster*. Fisher *et al.* (2012) identified increased levels of CG18135 RNA in both early (0-4 hours) and late (20-24 hours) embryos (Fisher *et al.*, 2012). Regarding other developmental stages, Casas-Vila *et al.* (2017) found highly increased levels of CG18135 RNA in pupae and in adult flies compared to embryos, suggesting the gene plays an important function beyond the embryo stage (Casas-Vila *et al.*, 2017). Moreover, it has been shown that one week old adult males have significantly higher *CG18135* gene expression than similarly aged females (Brown *et al.*, 2014; Casas-Vila *et al.*, 2017).

The aim of this study was to make a functional characterization of an insertional allele of *CG18135* gene (symbolized $CG18135^{P\{lacW\}CG18135}$) obtained in our laboratory. The main objectives of the study were: i. to investigate the viability of heterozygous and homozygous $CG18135^{P\{lacW\}}$ mutant flies during various developmental stages, and ii. to evaluate the *CG18135* expression level in mutant heterozygotes.

Materials and methods

In this study, two distinct *D. melanogaster* lines were used in order to investigate the viability and gene expression of *CG18135*: $CG18135^{P\{lacW\}CG18135}$ mutant line and the control Canton Special (CantonS) line. The mutant flies harbor at least one insertional allele caused by a *P{lacW}* insertion in the 5' intronic region of the *CG18135* gene (GenBank/NCBI accession number: HQ695001.1). The mutant individuals were maintained versus two distinct balancer chromosomes and the two derived lines were symbolized as it follows:

- y^-, w^-; $CG18135^{P\{lacW\}CG18135}$-*Sep1H* / TM3, *Ser*, *GFP*, *e*; in brief, $CG18135^{P\{lacW\}}$/GFP;
- w^-; $CG18135^{P\{lacW\}CG18135}$-*Sep1H* / TM6B, *Tb*, *Hu*, *e*; in brief, $CG18135^{P\{lacW\}}$/TM6B.

The flies were raised on wheat-banana-yeast-agar based medium at 19°C. All the experiments involving living flies were performed using the same standard medium, at room temperature.

I. Viability experiment

Flies from the $CG18135^{P\{lacW\}}$/GFP line (200 females and 100 males) and CantonS line (130 females and 65 males) were placed for an hour in 50 mm embryo collection cages coupled with solid medium Petri dishes. Throughout the developmental stages, the fluorescent phenotype, determined by the presence of the *GFP* construct, was assessed by using an Olympus U-RFL-T UV lamp.

II. Gene expression experiment

CG18135 gene expression was measured on groups of 50-hour old adult females and males from $CG18135^{P\{lacW\}}$/TM6B mutant line and CantonS control line. Biological triplicates consisting of 20 flies from each sex were treated for RNA stabilization using the DNA/RNA Shield kit, then submitted to RNA extraction using the RNeasy Mini Kit. cDNA conversion was performed combining the SuperScript II Reverse Transcriptase kit and Reverse Transcription System kit using random primers and adjusting the RNA volumes based on RNA concentrations.

qRT-PCR amplifications were performed in technical triplicates using the SYBR Green ROX qPCR Mastermix kit with custom *CG18135* and *RpL32* endogene primers (Figure 1). Each qRT-PCR reaction tube contained 20 ng of cDNA, 0.5 µM of primers (for each forward/reverse primer), 1X Mastermix solution and PCR-grade water up to 20 µL final reaction volume. The cycle threshold (Ct) values were gathered at a fluorescence threshold Rn = 0.2.

Gene	Orientation	Nucleotide sequence	Molecular mass (g/mole)	Melting temperature (ºC)	CG %
CG18135	F	5' ATCTTTCAGATCAGGCTGCC 3'	5987	55,8	50
	R	5' GACATAGGGAAGCCTCAGCC 3'	6136	57,5	60
RpL32	F	5' CGGCTTCAAGGGACAGTATC 3'	6102	62	55
	R	5' GACAATCTCCTTGCGCTTCT 3'	6019	60	50

Figure 1: Characteristics of the custom primers used for gene expression analysis with qRT-PCR. The primers' melting temperatures were provided by the manufacturers, Integrated DNA Technologies for *CG18135*, and Eurogenctec for *RpL32*.

Data analysis

Microsoft Excel was used for data manipulation of viability/lethality and analysis of fold change (FC). The latter data analysis was performed using the Livak formula (Livak and Schmittgen, 2001) on Ct values. For evaluating the statistical significance of gene expression variation we used $2^{-\Delta Ct}$ values. GraphPad Prism 8 was used for graphical representations and RStudio Desktop 4.2.0 for statistical analysis. Statistical significance was considered at a p value < 0.05.

Results

I. Viability experiment

We obtained a similar number of embryos for both evaluated lines, namely 246 from the *CG18135*$^{P\{lacW\}}$/GFP females and 251 from the CantonS females.

In this experiment, we evaluated the viability of the two compared lines by quantifying the number of embryos that do not reach the larval stage and, afterwards, the number of viable adults that emerged from pupae. For the CantonS embryos we observed a mortality rate of ≈ 8.8%, this value being considered a reference. The overall mutant embryo lethality was ≈ 21.5%, considerably higher than that recorded for the control. Considering that the embryos were the product of mating between *CG18135*$^{P\{lacW\}}$/GFP female and male individuals, theoretically, there were three possible embryo genotypes: *CG18135*$^{P\{lacW\}}$/GFP (50%), *CG18135*$^{P\{lacW\}}$/*CG18135*$^{P\{lacW\}}$ (25%) and GFP/GFP (25%). The latter category exhibits lethality during early embryonic stages, therefore we expected a total mutant embryo lethality of roughly 25%. In practice, statistical analysis (Figure 2A) showed no significant difference between the observed and expected embryo lethality for the descendants of the mutant *CG18135*$^{P\{lacW\}}$/GFP individuals ($\chi^2 = 0.64$ and $p = 0.423$ for the chi-square test, and $p = 0.394$ for the Fisher's exact test). Probably, among the dead embryos there were some pertaining to *CG18135*$^{P\{lacW\}}$/GFP and/or *CG18135*$^{P\{lacW\}}$/*CG18135*$^{P\{lacW\}}$ genotypes, but we believe that the majority of embryos were GFP/GFP.

Since monitoring the GFP fluorescence marker is a problematic task when one is trying to precisely assess the larval stages (because of larvae tendency to burry themselves within the medium), we counted the number of GFP (*CG18135*$^{P\{lacW\}}$/GFP) and nonGFP (mutant *CG18135*$^{P\{lacW\}}$/*CG18135*$^{P\{lacW\}}$ and CantonS control) pupae. Further, we recorded the number of adult individuals that hatched from each category. We did not observe any *CG18135*$^{P\{lacW\}}$/*CG18135*$^{P\{lacW\}}$ homozygous adults escapers emerging from 31 corresponding pupae, thus we considered a 100% lethality for the homozygous mutant pupae. As expected, the statistical analysis (Figure 2B) showed significant differences when comparing the lethality of homozygous mutant pupae to the 6.7% lethality of CantonS pupae ($\chi^2 = 142.03$ and $p = 2.2 \times 10^{-16}$ for the chi-square test, and $p = 2.2 \times 10^{-16}$ for the Fisher's exact test) or the 10.3% lethality of mutant heterozygous pupae ($\chi^2 = 90.731$ and $p = 2.2 \times 10^{-16}$ for the chi-square test, and $p = 2.2 \times 10^{-16}$ for the Fisher's exact test). The lethality of the mutant heterozygous pupae did not significantly differ from that of CantonS pupae ($\chi^2 = 0.855$ and $p = 0.355$ for the chi-squared test, and $p = 0.623$ for the Fisher's exact test).

The overall viability of the CantonS line (considered the reference viability) was defined as the percent of laid embryos that developed to the adult stage, which in our experiment was ≈ 72%. Assuming that about 50% of embryos have the *CG18135*$^{P\{lacW\}}$/GFP genotype, we would expect that their overall viability should be comparable to that of control embryos. Specifically, from the 246 mutant embryos, one should expect 88 heterozygous adult flies. The observed number of heterozygous mutant adult flies (n = 104) did not significantly differ from the expected number ($\chi^2 = 1.208$ and $p = 0.271$ for the chi-squared test, and $p = 0.241$ for the Fisher's exact test), suggesting that, indeed, the viability of *CG18135*$^{P\{lacW\}}$/GFP flies was similar to the control.

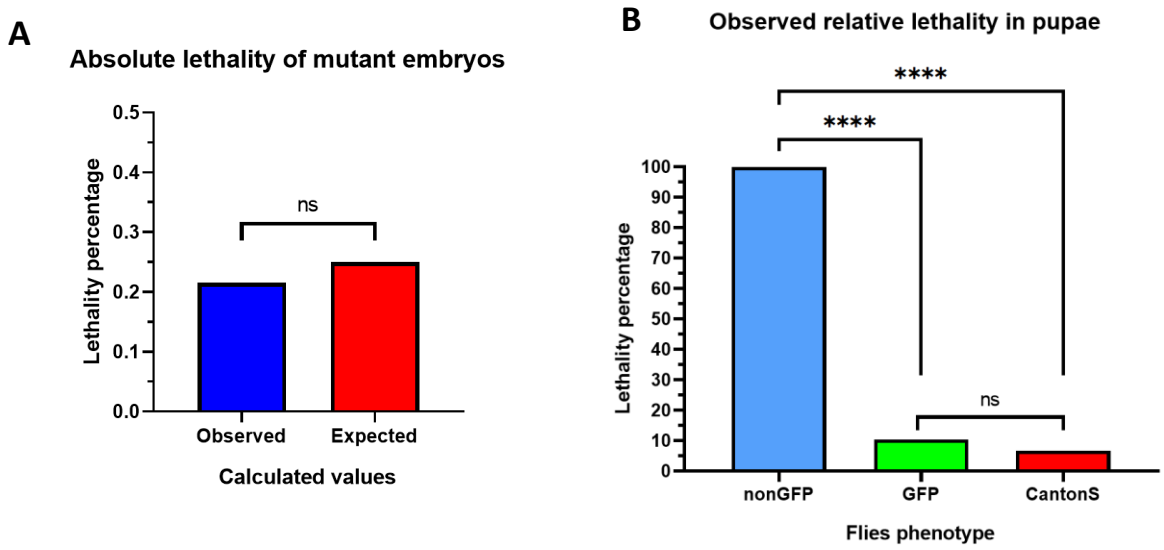

Figure 2: Barplots of relative lethality percentages corresponding to embryonic (A) and pupal (B) developmental stages. **A.** Observed total lethality of embryos from the mutant line compared to the expected 25% lethality. **B.** Observed relative lethality of mutant and CantonS pupae. GFP = $CG18135^{P\{lacW\}}$/GFP mutants, nonGFP = $CG18135^{P\{lacW\}}/CG18135^{P\{lacW\}}$ mutants, control = CantonS. Statistical significance was considered at $p < 0.05$ (ns > 0.05, **** < 0.0001).

II. Gene expression experiment

Relative gene expression analysis was based on comparing FC values that were generated by using the Livak method. Our experiment data (Figure 3) showed a significant overexpression of *CG18135* in $CG18135^{P\{lacW\}}$/TM6B mutant males compared to CantonS control males (FC = 1.77) and a not significant downregulation in $CG18135^{P\{lacW\}}$/TM6B mutant females compared to CantonS control females (FC = 0.975). Moreover, *CG18135* was significantly overexpressed in mutant males compared to mutant females (FC = 4.72), as well as in control males compared to control females (FC = 2.62).

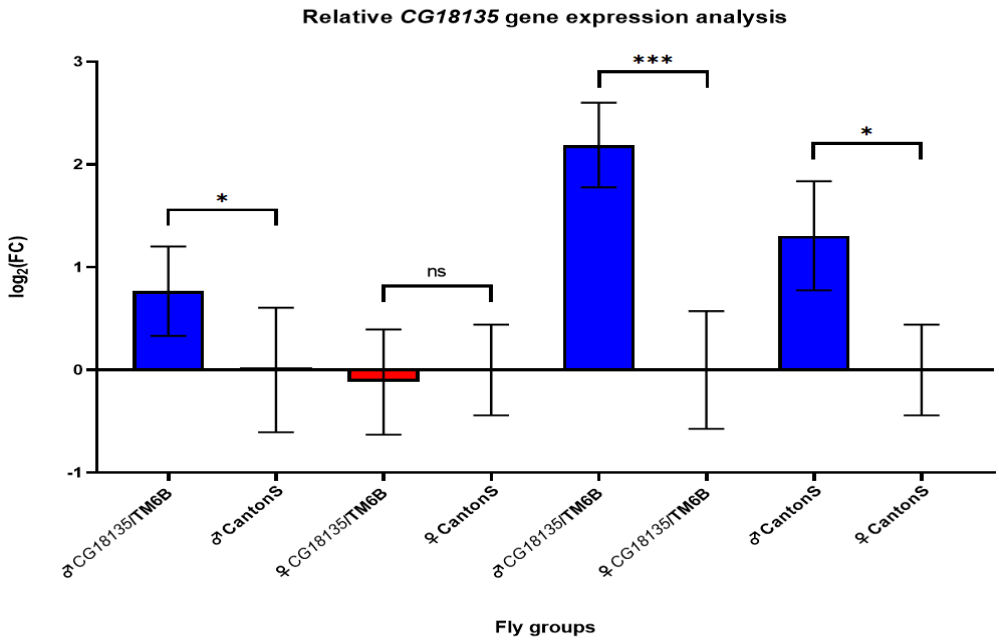

Figure 3: Relative gene expression analysis of CG18135 between mutant and control fly groups divided by sex criteria. The bar height represents $\log_2$FC values. Statistical significance was assessed using Student's t tests on $2^{-\Delta Ct}$ values and the critical value was considered at $p < 0.05$ (ns > 0.05, * < 0.05, *** < 0.001).

Discussion

I. Viability experiment

In this study we investigated the viability of *CG18135*$^{P\{lacW\}}$/GFP line offspring. We found that, for *CG18135*$^{P\{lacW\}}$/*CG18135*$^{P\{lacW\}}$ mutants, the pupal developmental stage was strongly associated with lethality. To our knowledge, this is the first viability analysis involving *D. melanogaster* individuals harboring a mutation in the *CG18135* gene.

The lack of statistically significant differences between observed and expected lethality of mutant embryos suggests that the *CG18135*$^{P\{lacW\}}$ allele is not associated with embryo lethality. This result could be attributed to the parental delivery of functional CG18135 proteins in either the unfertilized egg (Fisher *et al.*, 2012) or in the seminal fluid (Findlay *et al.*, 2008), thus fulfilling the CG18135 protein requirements in early embryonic stages.

In addition, we observed that none of the *CG18135*$^{P\{lacW\}}$/*CG18135*$^{P\{lacW\}}$ pupae developed to the adult stage. Previous proteomics studies identified increased CG18135 protein levels in *D. melanogaster* larvae, pupae and adults, compared to embryos (Brown *et al.*, 2014; Casas-Vila *et al.*, 2017). The CG18135 protein participates in myosin filament binding within muscular tissues, as well as in cell division (Liu *et al.*, 2008), and it is expected that the protein requirement increases during pupal development which implies organogenesis and high rates of cellular division. We hypothesize that in *D. melanogaster* the *CG18135* gene participates in various biological and molecular processes, similar to its orthologous gene in higher eukaryotes, *GPCPD1*. In mice, Okazaki *et al.* (2010) showed that *GPCPD1* gene expression was downregulated in atrophied skeletal muscles, and *GPCPD1* gene knock-out enhances myoblastic differentiation and limits the progression of skeletal muscle atrophy (Okazaki *et al.*, 2010). Thus, a lack of sufficient CG18135 protein could increase the lethality rate in pupae, as observed in our study.

High expression of *CG18135* was found in the Bolwig's organ (Fisher *et al.*, 2012), and, the corresponding protein was identified within the plasma membrane of *D. melanogaster* adults' heads (Aradska *et al.*, 2015). In humans, *GPCPD1* knockdown malignant cell showed an increased intracellular glycerophosphocholine/phosphocholine (GPC/PC) ratio, a decreased lipid metabolism and a cell migration inhibition (Steward *et al.*, 2011). In higher eukaryotes, GPCPD1 facilitates GPC degradation, an essential phospholipidic membrane constituent (Eibl, 1980; Okazaki *et al.*, 2010), into glycerol-3-phosphate (G3P) and choline (Steward *et al.*, 2012). In eukaryotes, the G3P requirement is obtained from glycolysis (Li *et al.*, 2019; Nguyen *et al.*, 2019) and, together with choline, it is further used for GPC synthesis via the Kennedy pathway (Veldhuizen *et al.*, 1989; Fagone and Jackowski, 2013). These observations suggest that the CG18135 protein may be involved in regulating a negative feedback loop between G3P, choline and GPC (Figure 4). Furthermore, choline contributes to *de novo* synthesis of phosphatidylcholine and phosphatidylethanolamine membrane constituents via the Kennedy pathway (Steward *et al.*, 2012; Gibellini and Smith, 2010), and participates in Krebs cycle (Steward *et al.*, 2012), being an essential component in energy metabolism.

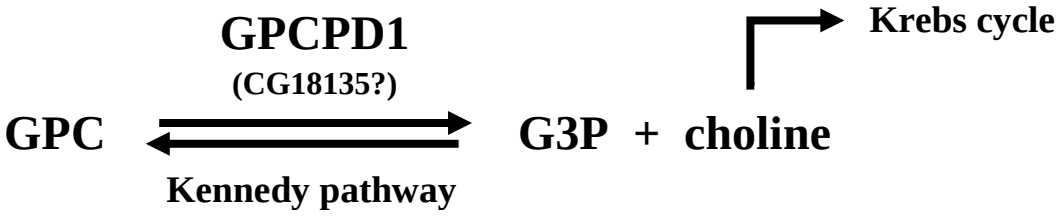

Figure 4: Biochemical pathway of GPC catabolism involving GPCPD1 protein (in humans) and possibly the CG18135 protein (in *D. melanogaster*).

II. Gene expression experiment

Considering the above results and hypotheses regarding the viability of *CG18135*$^{P\{lacW\}}$ mutant flies prior to adult developmental stage, we chose to evaluate the *CG18135* gene expression in adult flies, both in male versus female groups and mutant versus control groups.

Following the quantification of *CG18135* gene expression in different *D. melanogaster* adult groups, we found that *CG18135*$^{P\{lacW\}}$/TM6B mutant females and CantonS control females displayed a similar expression level, even if the mutant females had only one functional allele of *CG18135*.

Interestingly, we found that *CG18135* was significantly overexpressed in mutant males compared to control males. One possible explanation for this observation could be that, in the absence of one functional allele, there might be an increase in the GPC/PC ratio (Steward *et al*., 2012) which may result in a greater CG18135 protein requirement in order to reduce the GPC/PC ratio and, thus, an upregulation of *CG18135*. Nonetheless, because biochemical pathways involving phospholipids are complex, and because we did not observe a similar expression profile in mutant females compared to control females, a conclusion could not be drawn.

In males, it has been shown that the CG18135 protein is present in the seminal fluid of *D. melanogaster* individuals at mating (Findlay *et al*., 2008), which could partially explain the overexpression of *CG18135* gene in mutant or wild-type males compared to females. Additionally, the reproductive system of male flies is associated with intense cellular division and cellular differentiation during spermatogenesis (Fabian and Brill, 2012). These processes are dependent on glycerophospholipids availability and may influence the expression of *CG18135*.

The observed *CG18135* overexpression in males compared to female adult flies is consistent with previous transcriptomics studies (Brown *et al*., 2014; Casas-Vila *et al*., 2017). The *CG18135* overexpression in mutant males compared to mutant females (FC = 4.72) could be caused by a combination of factors that drive overexpression in mutant males compared to control males (FC = 1.77) and in control males compared to control females (FC = 2.62).

Conclusions

In this study we used *D. melanogaster* flies that harbor at least one copy of *CG18135*$^{P\{lacW\}}$ mutant allele. The viability experiment results suggest that the *CG18135* gene does not significantly influence the viability of homozygous embryos, but is strongly associated with lethality of mutant homozygotes in the pupal developmental stage. Whilst heterozygous flies develop apparently normal, the gene expression experiment revealed significant differences between *CG18135* expression in male compared to female flies and in mutant compared to control males. Our findings support further investigations of the *CG18135* gene in *D. melanogaster* for modelling the interactions of its orthologue in higher eukaryotes, *GPCPD1*.

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